REMARKS/ARGUMENTS

Status of the Claims

Applicants thank the Examiner for her assistance in this case and for the phone conference that took place on June 29, 2005. With the pending entry of this amendment, the status of the claims will be as follows:

Claims 1-4, 6, 12, 34-48 and 51-54 are withdrawn from consideration.

Claims 5, 7-11, 15-33 and 49-50 are cancelled.

Claims 13, 14 and 55 are currently amended herein.

Claim 56 was previously presented and is unchanged.

New claims 57, 58 and 59 have been added.

Thus, the pending claims currently at issue are claims 13, 14, 55 and 56-59.

Support for the new claims and the claim amendments is found in the specification. Neither the new claims nor the claim amendments introduce new matter. These amendments are made without prejudice and are not to be construed as abandonment of the previously claimed subject matter or acquiescence to any objection or rejection of record. Applicants respectfully request entry of the Amendment and reconsideration of the application in light of the amendments and arguments herein.

Election/Restriction

Applicants acknowledge that claims 1-4, 6, 12, 34-48 and 51-54 were withdrawn by the Examiner in the Office Action dated May 11, 2005, as allegedly directed to a non-elected invention. Applicants respectfully disagree, and reserve the right to prosecute these claims or similar claims in the future.

Claim Objections

The Examiner objected to claims 13 and 14 because of informalities. Applicants have followed the Examiner's suggestion and added the language *peptide consisting of the* to claims 13 and 14. Applicants respectfully request that this objection be withdrawn.

Double Patenting

The Examiner objected to claims 49 and 50 for alleged double patenting. Applicants respectfully disagree. However, solely for the purpose of advancing the prosecution of the present application, without acquiescing to the objection, and reserving the right to prosecute these or similar

claims in the future, Applicants have cancelled claims 49 and 50. The cancellation of claims 49 and 50 renders the Examiner's objection moot, and Applicants respectfully request that this objection be withdrawn.

35 U.S.C. §112, First Paragraph

Claims 55 and 56 were rejected under 35 U.S.C. §112, first paragraph. The Examiner alleges that claim 55(e), and thereby also 56(e), lack descriptive support. Applicants respectfully disagree. However, solely for the purpose of advancing the prosecution of the present application, without acquiescing to the rejection, and reserving the right to prosecute these or similar claims in the future, Applicants have amended claim 55 to remove subparagraph (e), thereby rendering the rejection moot.

35 U.S.C. §112, Second Paragraph

Claims 55 and 56 were rejected under 35 U.S.C. §112, second paragraph. The Examiner alleges that claim 55, subparagraphs (f) through (h) are allegedly incomplete for failing to describe essential structural relationships.

Applicants have followed the Examiner's suggestion and amended claim 55 to recite *SEQ ID NO: 2* in renumbered elements (e) through (g) for the purpose of removing any perceived ambiguity in the claim. Applicants respectfully request that this rejection be withdrawn.

35 U.S.C. §102

Claims 55 and 56 were rejected under 35 U.S.C. §102(b) as allegedly anticipated by Gray *et al.* (*Vet. Microbiol.*, 43:183-196 [1995]). More specifically, the Examiner states that the reference shows fragments of cytotoxin (e.g., figures 2 and 3 in that reference) that inherently have the properties of the cytotoxin fragments of claim 55(a). Applicants respectfully disagree. However, solely for the purpose of advancing the prosecution of the present application, without acquiescing to the rejection, and reserving the right to prosecute these or similar claims in the future, Applicants have amended claim 55.

Claim 55 previously recited an element in the opening lines of the claim, where the polypeptide comprises a fragment of the amino acid sequence of SEQ ID NO: 2, wherein the fragment is shorter than the full length polypeptide (*i.e.*, shorter than SEQ ID NO: 2). As previously written, this element applied to each subparagraph (a) through (h). Claim 55 has been rewritten where this element has been removed from the opening lines and applied individually to each of the subparagraphs (b) through (g).

Claim 55 subparagraph (a) has been amended to add an additional element. This element requires that the polypeptide of subparagraph (a) further comprises a subsequence heterologous (*i.e.*, not native to or of unrelated origin) to the *M. bovis* cytotoxin of SEQ ID NO: 2.

Support for this amendment to claim 55 subparagraph (a) is found in the specification. The expression vectors used in the present invention to express recombinant cytotoxin polypeptides result in the expression of fusion polypeptides comprising an in-frame 6xHis tag. These vectors are pProEXHTa (Life Technologies) used to express the 438-713 cytotoxin fragment (see the application at p. 50, line 29) and pT7-7 used to express the 643-927 and 590-927 cytotoxin fragments (see p. 51, line 30; and page 61, line 28). These 6xHis tag fusion expression vectors are known in the art. Attached at the APPENDIX are the manufacturer's product literature describing the pProEXHTa vector and a reference (*Acta Biochimica Polonica* 48(1):227-232 (2001)) describing the pT7-7 vector.

The cytotoxin polypeptides provided in amended claim 55 subparagraph (a) require a subsequence heterologous to the *M. bovis* cytotoxin of SEQ ID NO: 2. The specification teaches this limitation in the use of the pProEXHTa and pT7-7 expression vectors, which produce polypeptides comprising heterologous 6xHis fusion tags.

In order for a reference to anticipate an invention, the reference must teach each and every element of the claimed invention. Gray *et al.* (1995) uses *M. bovis* cytotoxin isolated from native sources. Thus, it is not possible for cytotoxin polypeptides as taught in Gray *et al.* (1995) to comprise any heterologous subsequences. Because Gray *et al.* (1995) does not teach every limitation of the claim, Gray *et al.* (1995) can not anticipate the claim.

Applicants have added new claims 57, 58 and 59, which are each dependent on claim 55. Claim 57 recites a limitation of the polypeptide of 55(a) where the heterologous subsequence is a 6xHis tag. Claim 58 recites a limitation of the polypeptide of 55 (b), (c), (d), (e), (f) or (g) where the polypeptide comprises a 6xHis tag. Claim 59 recites a limitation of the polypeptide of 55 (a), (b), (c), (d), (e), (f) or (g) where the polypeptide is a fusion polypeptide. These new claims are supported by the specification at the locations listed above where the use of 6xHis tag expression vectors pProEXHTa and pT7-7 are described.

Applicants have amended claim 55 in subparagraphs (e), (f) and (g). These subparagraphs now recite "a polypeptide comprising" the various fragments 438 through 713, 590 through 927 and 643 through 927 of SEQ ID NO: 2. Support in the specification for polypeptides with these particular amino acids has been discussed previously (see the Response filed March 25, 2004). Additionally, the specification describes polypeptides comprising these amino acids with the production of 6xHis tag fusion polypeptides (see the specification at p. 50, line 29; p. 51, line 30; and page 61, line 28).

In view of the current amendment, claim 55 is novel over the prior art, and Applicants respectfully request that this rejection be withdrawn. Furthermore, as claim 55 is novel, the new claims 57, 58 and 59 are also novel, and Applicants respectfully request that these new claims be entered into the record and passed to allowance.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance is respectfully requested.

If the claims are deemed not to be in condition for allowance after consideration of this Response, a telephone interview with the Examiner is hereby requested. Please telephone the undersigned at (510) 337-7871 to schedule an interview.

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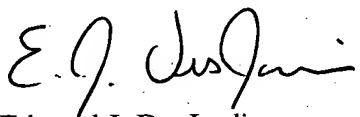
Respectfully submitted,



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Attachments:

1) APPENDIX with two references;
2) A petition to extend the period of response for **two** months;
3) A transmittal sheet; and
4) A receipt indication postcard.

GIBCOBRL

LIFE TECHNOLOGIES™

Product: **pPROEX™ HT Prokaryotic Expression System**
Cat. No.: 10711-018

Storage Conditions: 4°C
Size: 10 reactions

Description:
The pPROEX HT Prokaryotic Expression System is designed for the expression of foreign proteins in *E. coli* (1). The protein is expressed fused to a 6 histidine sequence $(His)_6$ for affinity purification. The gene of interest is cloned into the multiple cloning site (MCS) of either pPROEX HTa, pPROEX HTb, or pPROEX HTc. Upon expression, the histidine sequence is at the amino terminus of the fusion protein. The histidine sequence has a strong affinity for Ni-NTA resin matrix (2), making it simple to purify the desired protein (3). The vector also encodes the sequence for the Tobacco Etch Virus (TEV) protease cleavage site (4-8). rTEV Protease (Cat. No. 10127-017) can be used to remove the histidines from the fusion protein.

Component (see notes 1 and 2)	Part No.	Concentration	Amount
Plasmid pPROEX HTa	50609	0.5 µg/µl	10 µg
Plasmid pPROEX HTb	50610	0.5 µg/µl	10 µg
Plasmid pPROEX HTc	50611	0.5 µg/µl	10 µg
Control DNA pPROEX HT-CAT	50613	1 ng/µl	15 ng
Ni-NTA Resin	Y02281		10 ml
Column			one

Plasmid pPROEX HTa, pPROEX HTb, and pPROEX HTc:
The three pPROEX HT vectors contain a 6x histidine affinity tag for ease of purification, a 7-amino acid spacer arm, the TEV protease recognition site for cleavage of the 6x histidines from the protein, and an extensive multiple cloning site. The DNAs differ from each other with respect to their reading frames relative to the 6x histidine affinity tag. The *trc* promoter and *lacI*^{q} gene enable inducible expression of a cloned gene with IPTG. The plasmids also contain the pBR322 origin of replication, the β-lactamase gene conferring ampicillin resistance (Ap^r), and the bacteriophage F1 origin of replication (F1 intergenic region) for the synthesis of single stranded DNA in conjunction with the appropriate helper phage (9). These vectors cannot be used for blue/white screening in the presence of X-gal.

Control DNA pPROEX HT-CAT:
The control DNA pPROEX HT-CAT contains the chloramphenicol acetyl transferase gene (CAT) cloned into the *Ehe* I site of the plasmid pPROEX HTa. To verify that your expression system is operating properly, transform 100 µl of SUBCLONING EFFICIENCY™ DH5α™® Competent Cells (Cat. No. 18265-017) with 5 µl of the pPROEX HT-CAT plasmid according to the protocol supplied with the cells. These transformants can be selected on plates containing LB + 100 µg/ml ampicillin.

Ni-NTA Affinity Resin (see notes 1-3 on page 4 for additional information):
The Ni-NTA resin is supplied as a 50% slurry in 30% ethanol. Protein purification using this resin is based on the principles of immobilized metal chelate affinity chromatography (8). The NTA (nitrilo-tri-acetic acid) ligand occupies four of the six ligand binding sites of Ni^{2+}, leaving 2 sites free for interaction with the histidines. The resin is reusable 3-5 times.

GROWTH AND INDUCTION OF RECOMBINANT pPROEX HT CLONES

Before cloning into the pPROEX HT expression vectors, determine which vector is appropriate so that your protein will be in the correct reading frame. Digest the pPROEX HT DNA and the gene of interest with the appropriate restriction endonucleases to preserve the reading frame of the protein, then ligate the DNAs. Transform SUBCLONING EFFICIENCY DH5α Competent Cells (Cat. No. 18265-017) or MAX EFFICIENCY® DH10B™ Competent Cells (Cat. No. 18297-010) with the ligated DNA (100 µl of cells per cloning reaction) following the procedure specified with the cells. Grow the transformants in LB + 100 µg/ml ampicillin overnight, and then isolate plasmid DNA using the standard mini-preparation procedure (11). Verify that the target gene has been cloned correctly before inducing the expression of the cloned gene as described below.

Small Scale Induction of Recombinant pPROEX HT:
1. Inoculate 2 ml of LB media + 100 µg/ml ampicillin with a single colony. Incubate the culture overnight at 37°C with agitation., see note 4.
2. The next day, inoculate 10 ml of LB media + 100 µg/ml ampicillin with 0.1 ml of overnight culture. Grow at 37°C with agitation., see note 4.
3. When the culture reaches an A_{590} of 0.5-1.0, remove 1 ml and centrifuge for 1 min in a microcentrifuge. Discard the supernatant and resuspend the cells in 100 µl of PBS (Cat. No. 10010-015). This will be the uninduced sample.
4. To the remaining culture, add IPTG to a final concentration of 0.6 mM and continue to incubate the culture as described above.
5. Remove 1-ml aliquots of cells 1, 2 and 3 h after induction and measure the A_{590}. Centrifuge the cells as above and resuspend the pellets in 100 µl of PBS. These will be the induced samples.
6. Place 0.2 A_{590} of each sample in a separate microcentrifuge tube and mix with an equal volume of 2X SDS sample buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue]. Boil the samples and analyze them by SDS-polyacrylamide gel electrophoresis.

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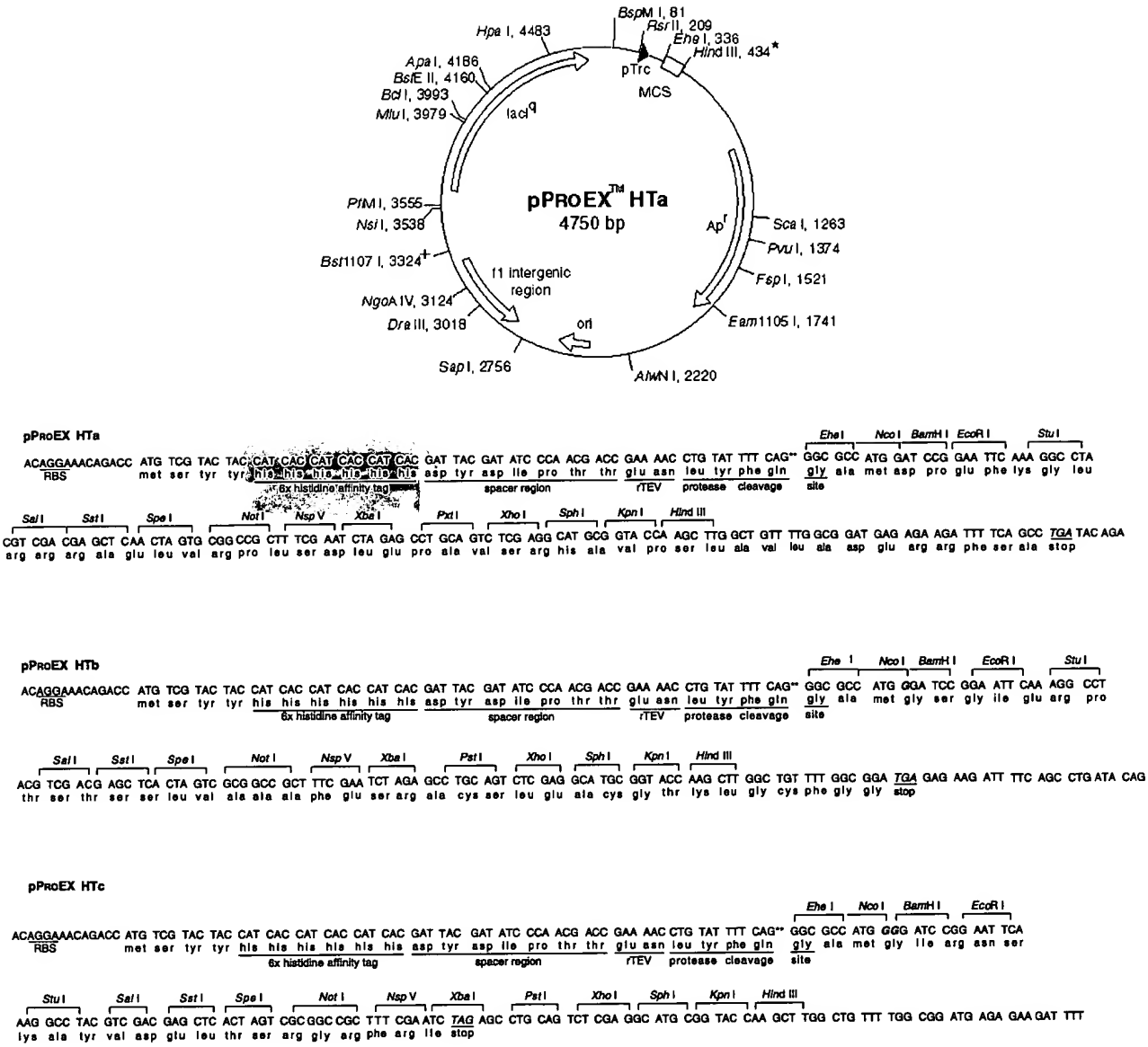

Figure 1. Map and multiple cloning sites of pPROEX HT expression vectors. The schematic of pPROEX HTa (4750 bp) with selected single digestion restriction endonucleases is presented. A similar schematic can be drawn for the other two vectors except that pPROEX HTb is 4778 bp and pPROEX HTc is 4779 bp. The additional bases for pPROEX HTb and pPROEX HTc reflect the insertion of a diagnostic restriction site at the *Bst* 1107 I site (+). In addition, the sites after *Hind* III (*) are shifted by +1 and +2 bases for pPROEX HTb and pPROEX HTc, respectively. The multiple cloning sites (MCS) for the 3 vectors are shown above. The sequence for the $(his)_6$, spacer region and recombinant TEV protease cleavage site are underlined. The cleavage with TEV protease occurs between the gln and gly and is signified by (**). The shift in reading frame occurs at the *Bam*H I site in each vector. The added base(s) are shown in bold. The stop codon for each vector is underlined and italicized. The M13/pUC Reverse 23-Base Sequencing Primer (AGCGGATAACAATTTCACACAGG) can be used to sequence clones. The 3'-end of the oligonucleotide is the AGG of the RBS.

***There are two *Sph* I sites in the vectors.

NOTE: The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The most up-to-date restriction endonuclease information can be found on the Internet at www.lifetech.com.

Large Scale Induction of Recombinant pPROEX HT:

Using the optimal induction times determined above, scale up the procedure for purification of the protein as follows:

1. Inoculate 10 ml of LB media + 100 µg/ml ampicillin with a single colony. Incubate the culture overnight at 37°C with agitation.
2. The next morning, inoculate 500 ml of LB media + 100 µg/ml ampicillin with 5 ml of the overnight culture. Incubate the culture at 37°C with agitation.
3. When the culture reaches an A_{590} of 0.5 - 1.0, remove a sample prior to induction to serve as an uninduced control. Induce the remaining culture with IPTG (to 0.6 mM) and continue to incubate the culture at 37°C based upon the optimal time determined above.
4. At end of the induction period centrifuge the cells at 10,000 x *g* for 10 min. Decant the supernatant fluid, determine the wet weight of cells, and store at -70°C until ready for protein purification.

PURIFICATION OF 6X HISTIDINE AFFINITY-TAGGED PROTEINS

The expressed protein can be purified with either a Tris-based buffer system or a phosphate-based buffer system. In general, proteins purified with the Tris-based system have fewer contaminants. However, if your protein is sensitive to pH > 8.0, the phosphate system can be used with satisfactory results. Because the pH of Tris buffers vary with temperature, **the pH of the Tris buffer system must be determined at the temperature at which the column will run.** Phosphate buffers are not as sensitive to temperature.

- Do not use DTT in buffers. This chemical will reduce the Ni^{2+} ions.
- Do not use EDTA or other chelating agents in buffers. These chemicals will chelate the Ni^{2+} ions.
- When analyzing samples on SDS-polyacrylamide gels, fractions containing imidazole should be heated to 37°C for 10 min instead of boiling. This will avoid imidazole mediated cleavage of labile peptide bonds.

Preparation of Extracts

1. Resuspend the cells in 4 volumes of lysis buffer [50 mM Tris-HCl, (pH 8.5 at 4°C), 5 mM 2-mercaptoethanol, 1 mM PMSF], see note 5.
2. Sonicate the suspension until 80% of the cells are lysed. Remove 500 µl and save. Label sample as "crack", see note 6.
3. Remove the cell debris by centrifugation.
4. Transfer the supernatant fluid to new tube. This is your CRUDE SUPERNATANT.
5. Save the pellet, see note 7.

Affinity Chromatography

If using a Tris buffer:

1. Equilibrate the column with Buffer A [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol, 20 mM imidazole) [see note 8].
2. Maintain a flow rate of 0.5 ml/min.
3. Load the sample from step 4 (the crude supernatant) on the column.
4. Wash the column with 10 volumes of Buffer A.
5. Wash the column with 2 volumes of Buffer B [20 mM Tris-HCl (pH 8.5 at 4°C), 1 M KCl, 5 mM 2-mercaptoethanol, 10% glycerol]
6. Wash the column with 2 volumes of Buffer A.
7. Elute bound proteins with Buffer C [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol, 100 mM imidazole], see note 8.
8. Collect 0.5 volume fractions.

If using a phosphate buffer:

1. Equilibrate the column with Buffer D [50 mM potassium phosphate (pH 6.0 at 25°C), 300 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol].
2. Maintain a flow rate of 0.5 ml/min.
3. Load the sample from step 4 (the crude supernatant) onto the column.
4. Wash the column with 10 volumes of Buffer D.
5. Elute bound proteins with Buffer E [50 mM potassium phosphate (pH 6.0 at 25°C), 300 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, 100 mM imidazole].
6. Collect 0.5 volume fractions.

Quick (Batchwise) Purification Protocol

1. Prepare the Tris buffers A and C. Remember it is critical that pH of Tris buffer be pH 8.5 at 4°C.
2. Prepare the extract as described above.
3. Transfer 500 µl of the crude extract to a new tube.
4. Add 200 µl of a 50% slurry Ni-NTA resin pre-equilibrated with Buffer A, see note 9.
5. Mix the suspension continuously for 20 min at 4°C.
6. Centrifuge the suspension for 1 min and transfer the supernatant to new tube. This is the material that does not bind to the resin.
7. Add 1 ml of Buffer A to the resin.
8. Mix the suspension for 5 min at 4°C.
9. Centrifuge the suspension for 1 min and transfer the supernatant to new tube and save. This is the wash fraction.
10. Repeat Steps 7-9.
11. Elute the protein with 3×200-µl aliquots of Buffer C. Mix each aliquot with the resin for 5 min, then centrifuge the sample 1 min in a microcentrifuge tube. Transfer the supernatant to a new tube. This is the eluted protein fraction.

REGENERATION OF THE NI-NTA RESIN

The number of times the Ni-NTA resin can be reused depends on the nature of the samples that have been previously run on the matrix. For best results, only purify identical recombinant proteins 3-5 times with the same batch of resin. If the Ni-NTA resin will be used to purify a different recombinant protein, regenerate the used resin according to the following procedure. Additionally, if the resin changes in color from light blue-green to brownish gray, use fresh resin or regenerate it before using it again.

Wash the column in the following order with:

1. 2 volumes of 6 M guanidine hydrochloride/0.2 M acetic acid
2. 2 volumes of water
3. 3 volumes of 2% SDS
4. 1 volume of 25% EtOH
5. 1 volume of 50% EtOH
6. 1 volume of 75% EtOH
7. 5 volumes of 100% EtOH
8. 1 volume of 75% EtOH
9. 1 volume of 50% EtOH
10. 1 volume of 25% EtOH.
11. 1 volume of water
12. 5 volumes of 100 mM EDTA, pH 8.0
13. 2 volumes of water
14. 2 volumes of 100 mM $NiSO_4$
15. 2 volumes of water
16. 2 volumes of 6 M guanidine hydrochloride/0.2 M acetic acid.

Equilibrate the column in Buffer A or Buffer D, depending on the buffer system to be used with your recombinant protein.

Quality Control:
This product has passed the following quality control: Verification of restriction endonuclease sites in the MCS and vector. pPROEX HT-CAT Control DNA is tested to ensure the DNA transforms DH5α. Binding capacity of Ni-NTA resin for a His tagged protein is verified at 4-8 mg per 1 ml of resin.

Notes:

1. Vectors and resin are manufactured for Life Technologies® by QIAGEN Inc.
2. This kit is provided with a license for research use only. Information in respect of licenses to use the products contained in this kit for purposes other than research may be obtained from F. Hoffmann-La Roche Ltd., Corporate Licensing, 4002 Basel Switzerland.
3. Additional Ni-NTA resin may be purchased from QIAGEN Inc., 9600 De Soto Ave., Chatsworth, CA 91311. (800-426-8157).
4. Lowering the temperature to 20°C-30°C may help stabilize the induction of a given clone and result in higher protein yield. Better induction results are generally obtained with fresh bacterial cultures. Inoculation of a culture from a plate that is several days old may give low yield of protein upon induction.
5. Add freshly-made PMSF to the solution since PMSF loses effectiveness within 30 min of dilution into an aqueous solution.
6. To calculate % cell lysis, dilute 5 µl of cell suspension prior to sonication in 995 µl of water and measure the absorbance at 590 nm (= $A_{initial}$). Sonicate the cells and dilute 5 µl of sonicated cells in 995 µl of water and measure the absorbance at 590 nm (= A_{final}). The proportion of cells lysed is given by

$$\% \text{ cells lysed} = 1 - (A_{final} / A_{initial})$$

7. Some proteins may be insoluble after induction. To determine if a protein is insoluble, resuspend the pellet (~5-10 O.D. of cells) in 1 ml lysis buffer. Sonicate to lyse cells and centrifuge for 2 min to pellet cell debris. Decant the supernatant and save on ice (this represents the soluble protein fraction). Resuspend the pellet in 1 ml lysis buffer (this is the insoluble protein fraction). Mix (15-25 µl) samples with an equal volume of 2X protein loading buffer and electrophorese samples on an SDS-polyacrylamide gel.
8. The salt concentration can be increased to 500 mM to improve protein purity.
9. The resin is equilibrated as follows: Mix the resin in the bottle well and pipet 1 ml of the slurry into a microcentrifuge tube. Centrifuge the resin for 30 sec and remove the supernatant. Add 500 µl equilibration buffer (either buffer A or buffer D) and mix well. Allow resin to incubate on ice for 5 min. Re-centrifuge resin and remove supernatant. Add 500 µl equilibration buffer and mix well. Resin is now ready for use.

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227-232

QUARTERLY

Expression of bovine leukemia virus protein p24 in *Escherichia coli* and its use in the immunoblotting assay✪

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Key words: bovine leukemia virus, *gag* gene cloning, fusion protein p24, immunoblotting assay

The *gag* gene encoded protein, p24 of bovine leukemia virus (BLV), was cloned and expressed as thioredoxin-6xHis-p24 protein in *Escherichia coli*. The bacterial cells carrying plasmid pT7THis-p24 expressed the protein of 38 kDa that was detected by immunoblotting analysis using anti-p24 monoclonal antibodies and sera from BLV infected cattle and sheep. The purified p24 fusion protein was shown to be sensitive and specific for detection of BLV antibodies in the infected cattle.

Enzootic bovine leukemia (EBL) is an infectious lymphoproliferative disease of cattle, caused by the retrovirus: bovine leukemia virus (BLV). Like other complex retroviruses the BLV genome contains the *gag, pol* and *env* structural genes and regulatory genes [1]. Most of the structural proteins of BLV are immunogenic but the naturally infected animals develop antibodies to *env*-encoded glycoproteins gp51 and gp30 as well as to *gag*-encoded proteins p24 and p15 [2]. The antibodies against p24 and gp51 are predominant [3]. BLV protein structure studied with monoclonal antibodies revealed the presence of three major conformational epitopes, F, G and H in the N-terminal region of gp51 and two epitopes in C-terminal region of p24 [4].

Since the presence of antibodies to BLV is a constant and early feature of BLV infection, serological examination of cattle sera is the best method for detection of infected animals. Most commonly used serological tests are the agar gel immunodiffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). However, variable results have been obtained by the two methods using the p24 or gp51 antigen. The disparities were often the result of differences in specificity

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Abbreviations: BLV, bovine leukemia virus; FLK, foetal lamb kidney; IPTG, isporopyl-1-thio-D-galactoside.

and sensitivity of the tests used. AGID is less sensitive and is not useful for detection of antibodies to p24 antigen. ELISA has been shown to detect both anti-gp51 and anti-p24 antibody with equal sensitivity but this method is prone to generate non-specific reactions. While in the ELISA the non-specific reactions are difficult to distinguish from specific ones, Western blot analysis allows precise resolution of the two reactions. So far limited studies have been performed to confirm the usefulness of the immunoblotting assay in the routine serological detection of BLV antibodies [5, 6]. Recently, the recombinant viral proteins have been found to be widely applicable in immunoassays for detection of specific antibodies. In particular, the use of the recombinant proteins synthesized in *E. coli* has been well documented in retroviral serology [7, 8].

In this study, the *gag* gene of BLV which encodes protein p24 was cloned and expressed as thioredoxin-6xHis-p24 fusion protein in *E. coli*. The recombinant p24 fusion protein was tested for sensitivity and specificity in detection of antibodies in sera of BLV-infected cattle by Western blotting assay.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmid pAMP1 and *E. coli* strain DH5α were purchased from Life Technologies. The pT7THis expression vector is a derivative of pT7-7 vector (USB) which contains the sequence encoding the *E. coli* thioredoxin (Trx) gene and 6 histidine residues (6xHis). This sequence is under control of the T7 RNA polymerase promoter. pT7THis is modified by insertion of *E. coli* Arg-tRNA gene into *Bgl*II site to allow the expression of eukaryotic genes rich in AGG and/or AGA codons. The multiple cloning site at 3′-end of 6xHis sequence allows the insertion of a DNA fragment fused in phase with ATG start codon of the *trx* gene sequence.

Cloning the gag gene in the expression vector. The preparation of plasmid DNA, DNA restriction, agarose gel electrophoresis, cloning and transformation of *E. coli* were carried out according to Sambrook *et al.* [9]. The *gag* gene (678 bp) encoding the protein p24 was amplified using forward primer 5′-CCAATCATATCTGAAGGGAA-3′ and reverse primer 5′-CAGAAGTGCAGGCTGTTTCA-3′ complementary to the BLV provirus sequence previously published [10]. In the 5′-ends of the primers, the extensions of dUMP residues and *Eco*RI and *Bam*HI recognition sites were added to the forward and reverse primers, respectively, for further cloning and subcloning purposes. DNA isolated from FLK cells infected with BLV (kindly provided by Dr J. Miller, NADC, Ames, IA) was used as the template. The amplified fragment was cloned into pAMP1 vector using the CloneAmp System (Life Technologies) according to manufacturer's instructions. The sequence of the insert was confirmed by the di-deoxy termination sequencing (Sanger). The recombinant plasmid pAMP1 containing the p24 encoding sequence was digested with *Eco*RI and *Bam*HI and the insert was ligated into the pT7THis expression vector digested with the same enzymes and transformed to *E. coli* strain B BL21 (DE3). The resulting plasmid pT7THis-p24 which, under the control of the T7 promotor, expressed thioredoxin and a short His tag fused to the N-terminus of full-length p24, was isolated. The p24 expression was regulated by *lac* repressor produced by *lacI* gene in *E. coli* host cells.

Isolation and detection of fusion protein. The *E. coli* BL21 (DE3) carrying plasmid pT7THis-p24 was grown at 30°C in LB medium [9] containing 100 μg ml^{-1} of ampicillin. When the culture had reached an A_{600} of 0.6 the expression of p24 was induced by addition of 1 mM IPTG. After 16 h of induction the cells were collected, sonicated and proteins were extracted with loading buffer (125 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate, 9% glycerol, 0.7 M 2-mercaptoethanol, 0.002% bromophenol blue). The soluble fraction of cell proteins was subjected to SDS/PAGE analysis [11]. The p24 fusion protein was purified by metal affinity chromatography on a Chelating Sepharose Fast Flow column (Pharmacia). The 6xHis-tagged protein was eluted with linear gradient of imidazole (0–0.25 M). In the immunoassay, the purified fusion protein p24 was transferred to

nitrocellulose membrane (Hybond-C, Amersham) by electroblotting and incubated on the filters with monoclonal antibodies anti-p24 of BLV (BW5C6H11 clone, CER, Belgium) or with bovine and sheep sera from BLV-infected and BLV-negative animals. The immunoassays were carried out as described previously [12].

Western blot analysis of sera from BLV infected cattle. Twenty two serum samples derived from cattle of different clinical status (lymphosarcoma, persistent lymphocytosis, and aleukaemic cattle showing no clinical symptoms) were tested by immunoblotting with the purified recombinant p24 fusion protein or, with culture supernatant- derived semipurified BLV virions [12] as test antigen. The results were compared to those obtained in the commercial ELISA (Synbiotics, France) and AGID (Dr Bommeli, Switzerland).

RESULTS

A 642 bp fragment of the *gag* gene containing the entire encoding sequence of the 24 kDa protein of BLV was amplified and inserted into vector pAMP1 by using a ligation-independent cloning system. The plasmids isolated from single white colonies of Amp^r transformants were screened for the correct insert by restriction analysis using *Eco*RI and *Bam*HI enzymes. Sequence analysis of the inserted fragment in the recombinant plasmid pAMP1.2 revealed 100% homology with the corresponding *gag* gene sequence of BLV (GenBank Accession No. M10987). The *Eco*RI/*Bam*HI fragment containing the *gag* gene encoding protein p24 was then inserted into the expression plasmid pT7THis. The single colonies of Amp^r transformants of *E. coli* BL21-DE3 were screened for the presence of the recombinant plasmids using restriction analysis, and the plasmid designated pT7THis-p24 containing the *Eco*RI/*Bam*HI insert of 654 bp was identified. The *E. coli* clone containing pT7THis-p24 expressed the fusion protein thioredoxin-6xHis-p24 following IPTG induction. The presence of this protein in the cell lysate was demonstrated by SDS/PAGE analysis (Fig. 1A, lane 1). After purification by metal-chelate affinity chromatography, the fusion protein migrated consistently as a single band with the molecular mass of 38 kDa (Fig. 1A, lane 3). The molecular mass of p24 was increased to 38 kDa with the 14 kDa thioredoxin-6xHis moiety at the N-terminus. The purified fusion protein was repeatly obtained at 55 mg/L of the *E. coli* culture.

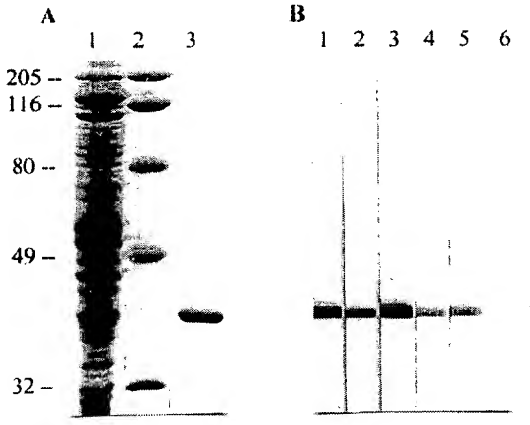

Figure 1. Characterization of BLV recombinant fusion protein p24.

(A) The extract of *E. coli* BL21 pT7THis-p24 total proteins induced with IPTG (lane 1); fusion protein Trx-6xHis-p24 purified by metal affinity chromatography (lane 3) separated by 10% SDS/PAGE as described by Laemmli [11] and stained with Coomassie blue. Positions of molecular size markers (lane 2) are given (in kDa) to the left of the figures (B) The purified fusion protein p24 separated by SDS/PAGE, transferred to Hybond C filter and incubated with: anti-p24 monoclonal antibody diluted 1:2000 (lane 1); BLV-infected cattle sera diluted 1:100 (lanes 2–4), BLV-infected sheep serum diluted 1:200 (lane 5) and BLV-negative cattle serum diluted 1:100 (lane 6). The colorimetric reaction was developed after incubation with peroxidase-labelled anti-species IgG according to the procedure described earlier [13].

To analyze BLV-specific immunoreactivity of the p24 fusion protein we performed Western blot analysis (Fig. 1B). Anti-p24 monoclonal antibodies (Fig. 1B, lane 1), bovine sera from BLV infected cattle (Fig. 1B, lanes 2–4) and serum from sheep experimentally infected with BLV (Fig. 1B,

lane 5) specifically and efficiently recognized the 38 kDa fusion protein. The intensity of the reactions varied between the antibodies used in the assay, with the monoclonal antibody and sheep serum being the most reactive, while the cattle sera were somewhat less reactive. No reaction was observed between the p24 fusion protein and serum from non-infected cattle (Fig. 1B, lane 6). The immunoreactivity of fusion protein p24 was highly specific, as demonstrated by the absence of cross-reaction of BLV-positive sera with thioredoxin moiety expressed in *E. coli* (not shown).

After evaluation of the BLV specific nature of p24 fusion protein, we determined whether the *gag* encoded protein could be used for the detection of anti-BLV p24 antibodies in sera from infected animals. Twenty two bovine serum samples were tested for the presence of BLV antibodies by ELISA and AGID. The results were compared to those obtained from Western blot analyses based on the recombinant protein p24 as antigen. Both ELISA and AGID detected only anti-gp51 antibodies. When the sera were tested by AGID, 18/22 were positive and 4 were negative. The ELISA tests detected anti-p24 antibodies in 20/22 with two doubtfull reactions. The latter two sera were also negative by the AGID assay. When the immunoblotting analysis was used, specific antibodies were found in all sera tested. All sera from clinically and haematologically positive cattle were found to be positive in all tests; inconsistent results were found only for sera from serologically positive animals.

In the next experiment we tested whether the immunoblotting assay with recombinant fusion protein p24 could be used as a confirmatory test for both AGID and ELISA. Four sera that were negative by AGID and gave inconsistent results by ELISA were tested with the blotted antigen p24, and all were found positive (Fig. 2, lanes 5–8). When these sera were tested by immunoblotting with the semi-purified virus from FLK-BLV cell culture supernatant as an antigen the antibodies anti-p24 were detected, however, anti-gp51 antibodies were detected weakly or not at all (Fig. 2, lanes 1–4). The lack of reactivity with gp51 correlated with AGID-negative and ELISA- doubtful reactions.

DISCUSSION

The use of conventional serological assays in BLV diagnosis still give inconsistent results, predominantly false-positive or negative reactions. Western blot immunoassay has been suggested as a suitable confirmatory test [6, 13]. However, the use of an appropriate BLV antigen seems to be crucial for the usefulness of this method [14]. The discrepancy between the results obtained in the conventional serological and in the Western blot assays regarding the gp51-binding antibodies has been reported previously [15, 16]. Although in sera from the infected animals the reaction with *gag*-related protein p24 is very clear, the reaction against gp51 may be weaker or absent. The lack of this reactivity in Western blot analyses, noted in our study, could be explained by the loss of conformational epitopes (F, G, H) of gp51 following disruption by the denaturing conditions of SDS/PAGE analysis [17]. The presence of bovine serum albumin and the insignificant amount of glycoproteins in the antigen preparation were

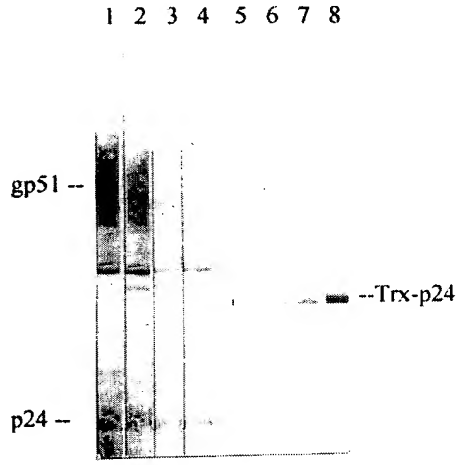

Figure 2. Western blot analysis of sera from BLV-infected cattle using different antigen preparations.

A sucrose gradient-purified BLV virus from FLK-BLV cell culture supernatant (lanes 1–4) and the purified fusion protein Trx-6His-p24 (lanes 5–8) separated by 10% SDS/PAGE were transferred to a Hybond C filter. The filters were incubated with the four sera which had given inconsistent results by the ELISA and AGID, and were subjected to Western blot analysis as described previously [12].

also considered as the factors contributing to the lack of immunostaining of gp51 in Western blots [14]. While in naturally infected animals anti-p24 antibodies were found less frequently and at lower titres than anti-gp51, their detection by Western blot analysis was consistent, despite the use of crude BLV antigen containing both p24 and gp51 [6, 13]. This was in accordance with our Western blot analysis using both viral and recombinant fusion antigens, although the immunostaining with the latter was more clear.

In conclusion, the immunobloting assay with recombinant p24 fusion protein as an antigen, is an efficient confirmatory test for the presence of specific antibodies in sera samples from BLV infected animals. Particularly in these cases when the animals may be considered negative because lack of the reaction with gp51. The fusion protein Trx-6xHis-p24 is expressed in *E. coli* as a soluble form at high concentration, circumventing the inefficient production of the retroviral antigens from the cell cultures. Furthermore, the detection of anti-p24 antibodies could potentially distingish the naturally infected from vaccinated animals, if BLV envelope glycoproteins were used as an immunogen.

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